Ricin: structure, mode of action, and some current applications

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ABSTRACT Ricin is an abundant protein component of *Ricinus communis* seeds (castor beans) that is exquisitely toxic to mammalian cells. It consists of an enzymic polypeptide that catalyzes the N-glycosidic cleavage of a specific adenine residue from 28S ribosomal RNA, joined by a single disulfide bond to a galactose (cell)-binding lectin. The enzymatic activity renders ribosomes containing depurinated 28S RNA incapable of protein synthesis. The bipartite molecular structure of ricin allows it to bind to the mammalian cell surface, enter via endocytic uptake, and deliver the catalytically active polypeptide into the cell cytosol where it irreversibly inhibits protein synthesis causing cell death. Because of its cytotoxic potency, modified ricin is being used for the selective killing of unwanted cells and for the toxigenic ablation of cell lineages in transgenic organisms.—Lord, J. M., Roberts, L. M., Robertus, J. D. Ricin: structure, mode of action, and some current applications. *FASEB J.* 8: 201–208; 1994.

Key Words: ricin · ribosome inactivating protein · lectin · depurination · endocytosis

RICIN IS A POTENTLY TOXIC PROTEIN found in castor oil plant (*Ricinus communis*) seeds. Many plants contain poisonous substances that have attracted the attention both of physicians, who have frequently found the sparing use of plant toxins to be therapeutically beneficial, and criminals, who have tended to be more liberal in the doses they use. Ricin fits readily into this classification; its anti-cancer potential, particularly as a component of immunotoxins, has attracted considerable attention recently, as did its more sinister reputed use in the assassination of the Bulgarian journalist Georgi Markov in London in 1978 (1).

The toxicity of *Ricinus* seeds has been recognized since ancient times. More than a century ago, Stillmark isolated a toxic protein from the seeds, which he termed ricin (2). Toxicity was believed to result from the observed ability of the ricin preparation to agglutinate red blood cells. More recent studies established that Stillmark's ricin preparations were a mixture of a potent cytotoxin (ricin) and a hemagglutinin (*Ricinus communis* agglutinin). Several workers, including Alexander Pihl, Sjur Olsnes, and their colleagues working in Norway, showed that the toxicity of ricin is due to its catalytic action on eukaryotic ribosomes. This work culminated in 1987 when Yaeta Endo and colleagues working in Japan identified the mechanism of action of ricin on 28S ribosomal RNA.

Since the isolation of ricin, many structurally and functionally related proteins have been characterized from a wide variety of higher plants. All these proteins are enzymes that specifically and irreversibly inactivate eukaryotic ribosomes such that the latter can no l nger participate in protein synthesis. Collectively known as ribosome-inactivating proteins (RIPs),[1] these proteins usually occur as monomers of approximately 30 kDa (called type I RIPs) and are frequently, but not always, N-glycosylated (3). In spite of their ribosome-inactivating activity, type I RIPs are not cytotoxic because they appear to have no means of entering eukaryotic cells in order to reach their ribosomal substrates. Indeed, certain plant tissues that are rich in type I RIPs, such as wheat germ or barley grain, are widely consumed by humans and animals. In certain plant tissues, however, the RIP is covalently joined through a disulfide bond to a second polypeptide, which in all cases described to date is a galactose-binding lectin whose molecular mass is also around 30 kDa. These heterodimeric toxins (type II RIPs) bind to eukaryotic cells by interacting with cell-surface galactosides, and after subsequently entering the cytosol are able to promote cell death by inhibiting protein synthesis. This group, including the toxic proteins abrin and modeccin, contains some of the most potent cytotoxins in nature. The most extensively characterized member of the group, ricin, is described in some detail here.

OCCURRENCE, STRUCTURE, AND BIOGENESIS

There are several isoforms of ricin including ricin D, ricin E, and the closely related lectin *Ricinus communis* agglutinin (RCA). Together they account for more than 5% of the total protein present in mature *Ricinus* seeds. Ricin is a heterodimeric type II RIP composed of a ribosome-inactivating enzyme (32 kDa, designated the A chain or RTA) linked t a galactose/N-acetylgalactosamine-binding lectin (34 kDa, the B chain or RTB) by a single disulfide bond. RCA in contrast is tetrameric, composed of two ricin-like heterodimers, each of which contains an A chain (32 kDa) and a galactose-binding B chain (36 kDa). In addition to their structural differences, these two proteins also differ in their biological properties. Whereas ricin is a potent cytotoxin but a weak hemagglutinin, RCA is only weakly toxic to intact cells but is a strong hemagglutinin. However, although RCA is only weakly toxic to intact cells in vivo, its isolated A chain is an RIP of comparable activity to ricin A chain in terms of its ability to modify ribosomes in vitro (4). The sugar binding specificities are also similar but not identical. Overall, however, ricin and RCA are closely related proteins and antisera raised against individual ricin A or B chains cross-

[1]Abbreviations: RiP, ribosome-inactivating protein; RTA, ricin toxin A chain; RTB, ricin toxin B chain; RCA, *Ricinus communis* agglutinin; TGN, trans Golgi network; ER, endoplasmic reticulum; BFA, brefeldin A; IT, immunotoxin; GVHD, graft-vs.-host disease; UAS, upstream activation sequence; FMP, formycin monophosphate; ts, temperature-sensitive; cs, cold-sensitive;

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The intracellular role of RTB in ricin cytotoxicity therefore may be to ensure delivery of RTA to a translocationally competent compartment, whereas the membrane translocation ability may be the exclusive property of RTA; however, the hypothesis is largely speculative at present. The potent cytotoxicities of many RTA-containing immunotoxins (ITs) (s e below) and the cytotoxic enhancement seen when some ITs are administered to BFA-treated cells (49) suggest that alternative intracellular routes of entry may be available. Ricin variants require at least one of the two RTB galactose-binding sites to remain functional for cytotoxicity, even when the toxin binds to an alternative surface receptor such as the mannose receptor of macrophages (40). This suggests that ricin may change binding sites from the surface mannose receptor to an intracellular galactosylated receptor during intracellular transport, and that this change is essential for cytotoxicity. Similarly, *Pseudomonas* exotoxin is taken into cells by endocytosis after binding to the α_2-macroglobulin receptor (50) but may have to interact intracellularly with the KDEL receptor to reach the cytosol (45). Clearly, much more work is required to elucidate the pathway normally used by that fraction of the endocytosed toxin that ultimately enters the cytosol. Theoretically the ER is an appealing compartment for toxin translocation (containing protein disulfide isomerase, molecular chaperones, and the membranous machinery for the transport of proteins and peptides, all of which ricin might exploit (47)), but as yet there is no direct evidence that this organelle is involved. Alternative transport pathways and sites of toxin translocation should also be considered. For example, ricin may leave the Golgi in vesicles that normally transport lysosomal enzymes bound to the mannose 6-phosphate receptor and RTA may translocate to the cytosol from a prelysosomal vesicle.

THERAPEUTIC APPLICATIONS OF RICIN

Ricin is prominent among a group of toxic proteins that have been used in attempts to selectively kill unwanted cells, in particular, malignant cells. Delivery to the target cell is achieved by linking the toxin to an antibody or growth factor that specifically or preferentially interacts with the target cell in question. Ricin has generally been linked to monoclonal antibodies by a disulfide bond, formed using heterobifunctional cross-linkers. Such conjugates are called ITs. Occasionally ricin holotoxin has been conjugated to the antibody, but m re often, to avoid nonspecific cell interactions mediated by RTB, the RTA subunit only has been used. Such conjugates display potent and specific cytotoxicity toward their target cells in vitro. However, when the same conjugates have been administered in vivo to tumor-bearing rodents or humans, the antitumor effects have often been disappointing (51).

In vitro applications

RTA-ITs have been used in vitro to purge bone marrow of unwanted cells prior to transplantation. In allogeneic bone marrow transplantation this has entailed using the IT to destroy T lymphocytes in the bone marrow taken from a hist compatible donor to reduce the incidence of graft-vs.-host disease (GVHD) in patients receiving the transplant (51). In autologous bone marrow transplantation, a sample of the patient's own bone marrow is removed before destroying the remainder. Before the patient can be successfully reinfused with the marrow removed previously, it must be free of malignant cells. Anti-T cell ITs have been used with some success to purge such bone marrow in the treatment of a variety of T cell leukemias and lymphomas (51).

In vivo applications

For in vivo use, RTA-ITs have generally proved more effective when the target cells are readily accessible to the bloodstream. In this regard, anti-T cell RTA-ITs have been the most successful, particularly in the treatment of steroid-resistant, acute GVHD (52). For the in vivo treatment of solid tumors, considerable problems can arise due to poor access of the IT to the tumor mass, lack of IT specificity, tumor cell heterogeneity, antigen shedding, breakdown or rapid clearance of the IT, and dose-limiting side effects. RTA-ITs cause vascular leak syndrome, resulting in hypoalbuminemia followed by weight gain and pulmonary edema (51). Repeated administration can be compromised by an immune response to both the antibody (often murine in origin) and the toxin components of the IT. Considerable effort has been and is being made to overcome these and other limitations. For example, early (or first generation) RTA-ITs contained RTA that had been chemically purified from *Ricinus* seeds. In addition to requiring rigorous purification procedures to completely exclude RTB contamination, the purified RTA was naturally glycosylated with mannose- and fucose-containing oligosaccharides. This allows the IT to interact with cells containing mannose and fucose receptors. In particular, the abundance of these receptors on liver cells led to the rapid hepatic clearance of the RTA-ITs. This problem was initially eliminated by chemically deglycosylating the RTA prior to IT construction (51), and more recently by the use of nonglycosylated recombinant RTA produced in *E. coli* (15). Recombinant DNA technology is increasingly used to produce recombinant ITs and other chimeric molecules for therapeutic use (46).

Although ITs have failed to live up to the high expectations initially placed on them and in spite of problems associated with their use at present, efforts currently under way should lead to further improvements and ensure that ITs will be increasingly useful as clinically important and selective cytotoxic regents.

TOXIGENIC ABLATION

Selective cell ablation is a useful way to investigate the developmental origin, fate, or function of particular cell lineages in an organism. Initially this was achieved by damaging or physically removing cells. Increasingly, toxigenic methods are being used that involve the expression of a toxic gene product in the target cells. This latter approach can overcome difficulties arising from either the failure to remove/destroy all target cells or general problems of cell accessibility that can sometimes limit the use of physical methods. Toxigenes are DNA fusions in which DNA encoding a potent toxin, such as RTA or a catalytically active fragment from a bacterial toxin such as diphtheria toxin, is placed under the transcriptional control of a tissue- or developmental stage-specific promoter and/or enhancer. When expressed intracellularly the toxigene product causes cell death. The introduction and expression of a toxigene in transgenic animals or plants may lead to cell type-specific ablation, which can be used to study developmental cell lineages or to generate animal models of degenerative diseases. Obviously, if the cell type selected f r ablation is crucial for survival it would be impossible t derive genetically modified strains through breeding. When the cell lineage is not essential for survival,

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Recombinant proricin binds galactose but does not depurinate 28 S ribosomal RNA

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Preproricin transcripts microinjected into *Xenopus* oocytes were expressed and the product was segregated by the oocyte endoplasmic reticulum and core glycosylated. Recombinant proricin was soluble, stabilised by intramolecular disulfide bonds and biologically active in that it could bind to immobilized lactose (selectin 2) or immobilized asialofetuin. Affinity-purified proricin did not catalyse the depurination of 28 S ribosomal RNA unless it was reduced, when slight but significant activity was observed. Gel filtration of the reduced proricin fraction showed that this depurination activity was not associated with proricin. The activity was apparently due to ricin A chain released by reduction from mature ricin which was, in turn, generated from proricin, presumably via endogenous oocyte endoprotease activity.

Proricin; Galactose binding; Depurination

1. INTRODUCTION

Ricin is a potently cytotoxic, heterodimeric protein found in the seeds of the castor oil plant, *Ricinus communis*. One polypeptide subunit (the A chain) is an enzyme which catalytically inactivates 60 S subunits of eukaryotic ribosomes and thereby causes cell death [1]. The second polypeptide (the B chain), which is covalently joined to the A chain by a single disulfide bond, is a galactose-specific lectin [1]. Recently, it has been shown that ricin A chain is an *N*-glycosidase which removes a specific adenine residue located within a highly conserved region of 26 S and 28 S ribosomal RNAs [2,3].

During its biosynthesis in *Ricinus* seeds, mature ricin is derived from a precursor – preproricin – by a series of contranslational and post-translational modifications during intracellular transport from the site of synthesis in rough ER, via the Golgi complex, to the site of accumulation within organelles termed protein bodies [4,5]. The preproricin polypeptide consists of a 35-residue N-terminal leader peptide followed by the mature A chain sequence which, in turn, is joined to the B chain sequence by a 12 amino acid linking sequence [6]. This linking sequence is proteolytically removed within the protein bodies [7].

There are, at present, no data available on the biological activity of ricin precursor polypeptides. Here we demonstrate that preproricin can be expressed by microinjecting in vitro-generated transcripts into *Xenopus laevis* oocytes. The expressed product was segregated into the oocyte endomembrane system, core glycosylated and the N-terminal signal peptide was removed. This in vitro expression system has previously been shown to segregate, process and fold efficiently recombinant ricin B chain into a soluble, biologically active confirmation [8]. Recombinant proricin has been purified from oocyte homogenates and its biological activity has been determined.

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Preparation and Characterization of Recombinant Proricin Containing an Alternative Proteas -Sensitive Linker Sequence

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The aim of this study was to determine the feasibility of utilizing a factor Xa-specific cleavage site within a recombinant protein containing the ricin A chain (RTA) sequence. Release of RTA is believed to be an essential step during the intracellular phase of ricin intoxication. Failure to incorporate such cleavage sites in fusions containing RTA results in a loss of toxin action (O'Hare, M., et al. (1990) *FEBS Lett.* 273, 200. Kim, J., and Weaver, R. F. (1988) *Gene* 68, 315). In this report we describe the introduction of a factor Xa-specific site in the linker of proricin, which we use here as a model substrate. Upon purification of the recombinant mutant proricin after expression in *Xenopus* oocytes, we demonstrate that the protease does have access to the engineered recognition sequence (albeit at low efficiency) and that the presence of the latter does not interfere with disulfide bond formation or the lectin activity of the ricin B chain moiety. Upon cleavage and reduction, the RTA polypeptide displays ribosome-inactivating ability, indicating that the presence of the modified linker at its C-terminus does not interfere with its catalytic activity. The general applicability of using such a cleavage site in recombinant fusions with RTA is discussed.

human Xa 44-46 kDa

INTRODUCTION

Ricin is a heterodimeric cytotoxin produced in the seeds of the castor oil plant, *Ricinus communis*. The mature toxin consists of a 32-kDa A chain (RTA) linked by a disulfide bond to a 34-kDa galactose-binding B chain (RTB) (*1*). Ricin intoxication of eukaryotic cells is initiated when the holotoxin interacts with cell surface glycoproteins and glycolipids containing galactose. This binding is mediated entirely by RTB, which is also believed to play a role in correctly transporting RTA during the ensuing endocytosis (*2, 3*). Membrane translocation of RTA, possibly from a Golgi compartment (*4, 5*), is then followed by catalytic inactivation of ribosomes in a step mediated entirely by reduced RTA. RTA is an RNA-specific *N*-glycosidase which acts on 28S or 26S rRNA leading to a specific depurination within a highly conserved region thought to be crucial in the interaction with elongation factors (*6*).

Ricin is synthesized in *Ricinus* seeds as a preproprotein which is converted to its mature form by a number of co- and post-translational modifications (*7, 8*). These begin as the protein is being segregated into the ER lumen and terminate after vesicular transport when the protein is finally deposited in protein body organelles where ricin accumulates. The initial preproricin molecule contains a 35 residue presequence (including a signal peptide), followed by RTA, a 12 amino acid residue linker, and the RTB sequence (*9*). The final processing steps include proteolytic removal of the linking peptide by enzymes contained within protein bodies (*8*). A study of the activities of the proricin precursor has shown that although it possesses sugar binding activity, it is unable to depurinate 28S rRNA (*10*). Catalytic activity is present only when peptide continuity between RTA and RTB is disrupted. Thus synthesis of RTA as an inactive proenzyme contributes to the safeguards which ensure that RTA does not inactivate endogenous plant ribosomes.

Ricin and RTA have been used extensively in a variety of conjugates designed for selective cell destruction (reviewed in ref 11). Conventionally, the toxin is linked to an antibody, lymphokine, or other protein entity by chemical means, which also introduces a reducible disulfide bond. Many such conjugates exert a potent cytotoxic effect upon their target cells. Increasingly, however, recombinant cytotoxic conjugates are b ing produced directly by expression of the relevant gene fusions. This has been a particularly successful approach with the bacterial toxins diphtheria toxin (DT) and *Pseudomonas* exotoxin A (PE). By fusing fragments of these toxins with alternative cell binding proteins such as α-melanocyte stimulating hormone (*12*), soluble CD4 (*13*), or a single chain Fv antibody fragment (*14*), cell-specific, single-chain cytotoxins have been generated. Similar single-chain fusions containing RTA are not cytotoxic (*15, 16*). It is believed that during intoxication, RTA must be released from its cell binding ligand to be competent for membrane translocation. Unlike DT and PE, RTA lacks a specific proteolytic cleavage site recognized by target cell proteases encountered upon cellular uptake. Introduction of a trypsin-sensitive sequence into an RTA–protein A (PA) fusion protein demonstrated for the first time that a noncytoxic fusion protein containing RTA could be converted into a cytotoxic conjugate (*16*).

In the present report we describe the production and characterization of a recombinant variant possessing an alternative protease-sensitive linker separating RTA from RTB. Using proricin as a model recombinant fusion, we reveal that, as in native proricin, the mutant precursor possesses lectin activity but has no RTA activity until treated with the appropriate protease and reduced. Specific cleavage generates disulfide-bonded subunits with the biological pr perties of native holotoxin. This particular arrangement of modified linker and flanking cysteines, which does not significantly perturb the structure of the component polypeptides, may have general applicability in creating disulfide-linked conjugates from single-chain recombinant polypeptides containing RTA.

EXPERIMENTAL PROCEDURES

Construction of Plasmids. Preproricin cDNA (*9*) was subjected to site-directed mutagenesis to create a mutant clone with a linker encoding a factor Xa recognition

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Human Immunodeficiency Virus Protease

BACTERIAL EXPRESSION AND CHARACTERIZATION OF THE PURIFIED ASPARTIC PROTEASE*

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The protease of human immunodeficiency virus has been expressed in *Escherichia coli* and purified to apparent homogeneity. Immunoreactivity toward anti-protease peptide sera copurified with an activity that cleaved the structural polyprotein *gag* p55 and the peptide corresponding to the sequence *gag* 128–135. The enzyme expressed as a nonfusion protein exhibits proteolytic activity with a pH optimum of 5.5 and is inhibited by the aspartic protease inhibitor pepstatin with a K_i of 1.1 μM. Replacement of the conserved residue Asp-25 with an Asn residue eliminates proteolytic activity. Analysis of the minimal peptide substrate size indicates that 7 amino acids are required for efficient peptide cleavage. Size exclusion chromatography is consistent with a dimeric enzyme and circular dichroism spectra of the purified enzyme are consistent with a proposed structure of the protease (Pearl, L. H., and Taylor, W. R. (1987) *Nature* 329, 351–354). These data support the classification of the human immunodeficiency virus protease as an aspartic protease, likely to be structurally homologous with the well characterized family that includes pepsin and renin.

The etiological agent of acquired immune deficiency syndrome (AIDS)[1] is the retrovirus human immunodeficiency virus (HIV). HIV proteins, like other retroviral proteins, are initially translated as the large precursor polyproteins *gag*, *pol*, and *env* and are proteolytically processed to generate structural proteins (p17 (MA), p24 (CA), p7 (NC), and p6), enzymes (protease, reverse transcriptase, and integrase), and the envelope proteins (gp120 (SU) and gp41 (TM)). Processing of the *gag* and *pol* polyproteins is believed to involve a virally encoded protease. Among the retroviral proteases that have been characterized are those from the avian myeloblastosis virus, bovine leukemia virus, and murine leukemia virus (2–5). Mutations within the protease coding regions of retroviruses have been shown to result in noninfectious virions (6), including the case of HIV-1 (7).

There is limited amino acid sequence similarity between the well characterized aspartic proteases (such as pepsin and renin) and the HIV protease. Based on this similarity which involves the peptide sequences that flank the critical aspartate residues of the pepsins (8), the retroviral proteases have been speculated to belong to the family of aspartic proteases. Whereas retroviral proteases range in size from 10 to 14 kDa, the aspartic proteases are commonly observed to have masses of 33–44 kDa. Observation of similarity in the amino acid sequence between the 2 domains of the pepsin family of proteases suggested they have evolved from a duplication of genes (9). The smaller retroviral proteases have thus been postulated to function as dimers in analogy to the 2-domain structure of the pepsin class (10, 11).

The proteolytic activity of the HIV-1 protease has been observed with *in vivo* self-processing of portions of the *pol* gene product following expression in microbial cells and intermolecular reactions performed with that product have been observed with microbially expressed *gag* p55 as substrate (12–15). Attempts have been made to purify the microbially expressed protease, and partially purified material was used to demonstrate inhibition of the *gag* p55 cleavage reaction by 1 mM pepstatin (15). In addition, while this paper was in preparation, the purification of the protease from virions was reported, although the purified material had no activity (16). In this report, we describe the expression of the protease as a 10-kDa nonfusion protein in *Escherichia coli* and its purification as a soluble, active enzyme to apparent homogeneity. The activity of the purified protease with peptide substrates and its inhibition by pepstatin is quantitatively described. Furthermore, we present preliminary characterization of the protease structure with gel filtration and circular dichroism.

EXPERIMENTAL PROCEDURES

Materials—Peptides used as substrates or in the generation of antisera were synthesized by the Merrifield solid phase synthesis method (17). The substrate protein, HIV-1 *gag* p55, was cloned and expressed in yeast.[2] Chemically synthesized protease was kindly donated by Drs. R. Nutt and D. Veber (Merck Sharp and Dohme). The mono S column and plasmid pKK233-2 were from Pharmacia LKB Biotechnology Inc., HIV-1 viral lysate from Cytotech (San Diego, CA), and pepstatin A from Sigma. All other chemicals were reagent grade from standard suppliers. Rabbit antisera were raised against the peptide corresponding to *pol* open reading frame residues 102–114 (protease residues 34–46) by established methods (18). Oligonucleotides were synthesized using an Applied Biosystems model 380A DNA synthesizer.

Cloning and Expression—Recombinant DNA procedures were performed as described by Maniatis *et al.* (19). The 5′ portion (*Hin*dIII to *Kpn*I) of the *pol* gene of the NY5 (20) strain of HIV-1 was subcloned into the *Hin*dIII/*Kpn*I site of M13 mp8 and mp9. A mutation was introduced by oligonucleotide-directed mutagenesis to create an *Nco*I

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[1] The abbreviations used are: AIDS, acquired immune deficiency syndrome; HIV-1, human immunodeficiency virus, type 1; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane. Abbreviations for the retroviral proteins have recently been recommended (1) and are in parentheses in the text.

[2] G. Vlasuk, unpublished experiments.

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Activity and Dimerization of Human Immunodeficiency Virus Protease as a Function of Solvent Composition and Enzyme Concentration*

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The activity of human immunodeficiency virus 1 (HIV-1) protease has been examined as a function of solvent composition, incubation time, and enzyme concentration at 37 °C in the pH 4.5–5.5 range. Glycerol and dimethyl sulfoxide inhibit the enzyme, while polyethylene glycol and bovine serum albumin activate the enzyme. When incubated at a concentration of 50–200 nM, the activity of the protease decreases irrev rsibly with an apparent first-order rate constant of 4–9 $\times 10^{-3}$ min^{-1}. The presence of 0.1% (w/v) polyethylene glycol or bovine serum albumin in the reaction buffer dramatically stabilizes enzyme activity. In the absence of prolonged incubation of the enzyme at submicromolar concentration, the specific activity of HIV-1 protease in buffers of either high or low ionic strength is constant over the enzyme concentration range of 0.25–5 nM, indicating that dissociation of the dimeric protease, if occurring, can only be governed by a picomolar dissociation constant. Similarly, the variation of the specific activity of HIV-2 protease over the enzyme concentration of 4–85 nM is consistent only with a dimer dissociation constant of less than 10 nM. We conclude that: 1) the assumption of a nondissociating HIV-1 protease is a valid one for kinetic studies of tight-binding inhibitors where nanomolar concentrations of the enzymes are employed; 2) stock protease solutions of submicromolar concentration in the absence of activity-stabilizing compounds may lead to erroneous kinetic data and complicate mechanistic interpretations.

The proteases of the human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2)[1] process the GAG and POL precursor polyproteins to produce the structural proteins and enzymes of these retroviruses. As a result of the importance of the protease in the infectivity of the virus and the observation that inhibition of HIV-1 protease catalytic activity halts the replication of HIV-1 (1–3), extensive inhibitor discovery programs are currently underway. The majority of efforts thus far have concentrated on substrate-based competitive inhibitors of the protease (4–6). Since the active site of the protease is composed of equal contributions from each of two identical subunits (7, 8), it has been suggested that inhibitors which bind to the inactive monomeric subunit (dissociative inhibitors) may also afford a route to inhibition by promoting dissociation of the active dimer (9).

Dissociation constants (K_D) of monomer formation have been reported for the HIV-1 protease to be 3.6 nM at pH 5 (9) and 50 nM at pH 7 (10) and for the HIV-2 protease, 85 μM at pH 4.5 (11). It is difficult for one to reconcile these drastically different and unexpectedly high K_D values even taking into consideration that different methods and solvent conditions have been employed and that the two proteases vary in primary structure (12). Many previous studies have been conducted with various buffer systems employing enzyme concentrations at or below the quoted dissociation constants for determination of kinetic and inhibition parameters. Thus, the above-mentioned K_D values, if correct, would necessitate a re-evaluation of interpretations of published kinetic data and inhibition constants. For these reasons, the pivotal issue which needs be delineated is whether the protease subunits indeed dissociate in commonly used buffers during the experimental time course of kinetic and binding studies.

The literature to date contains many different assay systems, but only a few indications (13) of the effect the various components used in these systems have on the HIV proteases. This lack of information has rendered comparing results from different laboratories difficult and sometimes misleading. The effect of pH may further complicate the action of chemical additives to the enzyme reaction mixture. In this report, we present an analysis of some of the components often employed in HIV protease systems to determine their effects on the activity of HIV-1 protease. We have also examined the specific activities of both HIV-1 and HIV-2 proteases as a function of enzyme concentration using the same method and conditions. Our kinetic data reveal that, under the assumption of rapid equilibrium, the K_D value for each protease falls far below the values reported (9–11).

EXPERIMENTAL PROCEDURES

Materials—HIV-2 protease (ROD isolate) was subcloned into the TRP expression vector previously described (14) to produce the 99-amino acid subunit. Isolations of both HIV-1 and HIV-2 protease were performed as before (15). Enzyme concentrations were determined by active site titration (6). The fluorogenic substrate, Arg-Glu-[5-(aminoethyl)aminonaphthalenesulfonate]-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Lys(4'-dimethylaminoazobenzene-4-carboxylate)-Arg was obtained from Molecular Probes, Inc, and the nonchromophoric substrate Val-Ser-Gln-Asn-(β-naphthylalanine)-Pro-Ile-Val was from Bachem. The peptides acetyl-Thr-Leu-Asn-Phe and Ser-Gln-Asn-(Phe[ψ-CH_2N]Pro)-Ile-Val-Gln were synthesized by standard solid-

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[1] The abbreviations used are: HIV-1 and HIV-2, human immunodeficiency virus types 1 and 2, respectively; BSA, bovine serum albumin; k^{app}, the first-order rate constant describing the decrease in activity of HIV-1 protease; K_D, equilibrium constant for the dissociation of the HIV protease dimer; K_i, inhibition constant; PEG, polyethylene glycol; HPLC, high-performance liquid chromatography.

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Rationale for the Use of Immunotoxins in the Treatment of HIV-Infected Humans

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The first step in the replication of human immunodeficiency virus (HIV) is selective binding of the envelope glycoprotein (gp120) to CD4 receptors on T cells or macrophages. After penetration in these cells, the genome of the virus is integrated in the human genome. HIV-infection causes depletion of CD4-positive cells resulting in a severe immunosuppression. It is believed that eliminating HIV-infected cells is crucial in limiting further reduction of CD4-positive cells and thus, preventing disease progression. The most commonly used drugs, such as zidovudine (AZT), appeared to be not completely effective. Therefore many investigators are searching for alternative treatment modalities. The use of immunotoxins (ITs) to eliminate HIV-infected cells is discussed.

ITs are chimeric molecules in which cell-binding ligands are coupled to toxins and can specifically eliminate undesired cells. The cell-binding carriers of anti-HIV ITs have been directed against different regions of the HIV envelope glycoprotein (gp120 and gp41) and surface antigens (e.g CD4, CD25). The ITs have been composed of different ribosome-inactivating proteins (RIPs) like pokeweed antiviral protein (PAP), Pseudomonas exotoxin (PE), Diphtheria toxin (DT), or ricin. In *in vitro* studies, several of these ITs have been shown to be effective and specific in killing acute and persistently HIV-infected cells. The ITs were effective at concentrations (ID50 range from 10^{-9} M to 10^{-12} M) that were not toxic to uninfected cells or cells without the antigen. The IT CD4(178)PE40, a fusion protein directed against the CD4 binding site of gp120, has been investigated in two *in vivo* trials. The results were disappointing considering the antiviral activity *in vitro*. This was thought to be due to the rapid clearance of the IT and the differential resistance of clinical HIV isolates. Use of a panel of ITs is likely to be more effective because multiple approaches cover the intrinsic variability of HIV and the presence of IT-resistant or latently infected cells, as well as the blocking presence of neutralizing anti-HIV antibodies and the immunogenicity of most ITs.

It may be possible to control the virus completely with a panel of ITs in combination with other antiviral or immunosuppressive agents such as RT inhibitors (e.g AZT), interferon α, or cyclosporine. More research will be necessary to develop such a combined therapy.

Keywords: CD4, Drug Targeting, HIV Therapy, Immunotoxins, Ribosome-inactivating Proteins

INTRODUCTION

Acquired immune deficiency syndrome (AIDS) is a disease characterized by profound immunosuppression. AIDS manifests as a number of opportunistic infections followed by degenerative diseases and neoplasms. In the presence of a non-functional immune system, these illnesses devastate the patient and the disease is fatal.

It is widely accepted that the human immunodeficiency virus (HIV) is the etiological agent responsible for AIDS (reviewed in Fauci, 1988;

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Bryodin, a single-chain ribosome-inactivating protein, selectively inhibits the growth of HIV-1-infected cells and reduces HIV-1 production

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Summary. Bryodin, a single-chain ribosome-inactivating protein (RIP) isolated from *Bryonia cretica* ssp *dioica* (cucurbitaceae), was found to selectively inhibit the growth of persistently HIV-1-infected T lymphoma cells (KE37/1) and human lung fibroblast when used in concentrations from 2–20 µg/ml. Uninfected KE37/1 cells remained unaffected at the same doses of bryodin. In addition, bryodin reduced HIV production in the surviving infected cells. Two isoforms of bryodin were purified by dye ligand chromatography. Both isoforms exerted the growth-inhibiting influence and reduced HIV production. Trichosanthin, another member of the RIP family, had similar inhibitory effects on the growth of HIV-1 infected cells and on HIV-1 production. Bryodin and trichosanthin were effective in about the same dose range. No selective effects for HIV-infected cells were observed with the RIPs gelonin and ricin.

Key words: Bryodin – Ribosome-inactivating protein – T lymphoma cells – Antiviral agent – HIV-1 infection

Introduction

Human immunodeficiency virus type 1 infects predominantly T cells and cells of the monocyte/macrophage lineage. Some HIV-infected T cells and monocytes appear to be resistant to the cytopathic effect of the virus in vivo and continue to produce virions, thus creating an expanding virus reservoir [10, 13, 14]. This has been considered to play a central role in the progression of HIV-associated diseases.

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To interfere with the increasing virus reservoir in HIV-infected persons, two major strategies may be followed: (1) to inhibit virus production and release from the reservoir cells and (2) to inhibit the growth of infected cells or even to selectively eliminate them. This seems theoretically to be more efficient than prevention of the infection of new target cells. Some possibilities for the first strategy have been suggested, such as interference with virus transcription, post-translational modification of viral proteins, or virus assembly [12, 18, 26, 28, 37, 43, 45]. However, the growth control of HIV-infected cells has not been addressed intensively. Typical alterations in the cells due to HIV infection and replication might provide a target for a therapy designed to attack the HIV-infected cells. HIV replication modifies the cellular membrane metabolism, alters the antigenic makeup by presenting viral antigens as gp120 and gp41 on the cell surface, and influences the second-messenger pathways [4, 16, 17, 19, 21, 32]. Detection of cytotoxic immune responses in HIV-infected individuals indicates that HIV-antigen-presenting cells are immunologically active and that mechanisms for eliminating HIV-infected cells can possibly be induced by immunization [31, 33, 36, 46]. A different strategy has been investigated to eliminate HIV-infected cells by a passive immunotherapy using immunotoxins directed against gp 120 of HIV [3, 44].

An alternative approach is to make use of the altered membrane structures in HIV-infected cells. Altered membrane structure may result in an altered permeability for certain substances; thus, it might be possible to introduce toxins preferentially into cells infected with and producing HIV [4]. Ribosome-inactivating proteins (RIPs) are plant toxins with a molecular weight of about 30000; they are known to remove the 4324 adenine of the 28S rRNA by *N*-glycosidase activity [5–7, 42]. RIPs are able to inactivate ribosomes in a less-than-equimolar ratio. There are two types of RIPs that inhibit the protein synthesis in cell-free systems roughly to the same extent [7, 39]. Type 1 toxins (e.g., pokeweed, PAP) exhibit a relatively low toxicity to intact cells compared with type-2 toxins. The enhanced toxicity of type-2 RIPs (e.g., ricin) is due to a lectin that is bound to the active "A" chain by a disulphide bond. The lectin or binding "B" chain is able to bind to the target-cell surface and triggers the uptake of the "A" chain into the cell. Because type-1 toxins lack the "B" chain, their uptake into the cells occurs by passive transfer through the cell membrane. Therefore, type-1 toxins seem to be good candidates for selective inhibition of cells with altered membrane properties inasmuch as they have no carbohydrate-specific membrane affinity that mediates the uptake into the cells irrespective of the permeability of the membrane.

Here we describe effects of bryodin, a type-1 RIP isolated from *bryonia cretica* ssp *dioica* (cucurbitaceae), on the growth and the virus production of HIV-1-infected cells. This RIP is compared with other type-1 RIPs including trichosanthin, which has been previously reported to inhibit HIV replication [24, 25], and to ricin, a type-2 RIP.

Materials and methods

Cells

Uninfected human T-lymphoma cells (KE37/1) and permanently infected K37/1 cells with HTLV-III$_B$ [35] were obtained from M. Popovic. Human fibroblastoid cells (LC5) were de-

prokaryotic and eukaryotic GAPDHs all have the same secondary structure and since the evolutionary distance (amino acid sequence similarity) between site 1 and site 2 of the same GAPDH is much greater than the distance between site 1 of prokaryotic and eukaryotic GAPDHs, this would indicate that the duplication that gave rise to the two NAD^+-binding sites 1 and 2 occurred before the divergence of prokaryotes and eukaryotes. Hence, the occurrence of introns in similar positions in the two sites implies that these introns existed in the progenitor GAPDH gene prior to the divergence of prokaryotes and eukaryotes, but some of these introns were subsequently lost from certain GAPDH species during evolution. The fact that one site has introns in the plant chloroplast (GapB, B4) and animal cytosolic (chicken, K6) genes, while its duplicated site has introns in plant (GapC, C4) and animal (chicken, K4) cytosolic genes gives strong support to this idea. We predict that, as more sequencing data are available, some GAPDH genes should have introns located between residues 20 and 30 in site 1 corresponding to C5/K5 in site 2 and between residues 120 and 125 in site 2 corresponding to B2/C3/K3 in site 1. Second, the introns always appear between, but not within, the structural domains of the NAD^+ binding region. This observation is consistent with the idea, as proposed by Blake (*20*), that introns might play important roles in bringing together small sequence units that encode potentially stabilizing secondary structures in the evolving protein.

The relationships between intron positions and the catalytic domain, which consists basically of nine-stranded antiparallel sheets and a long helical tail (*16*), is more difficult to interpret. However, based on the same lines of reasoning, Stone *et al.* (*19*) have argued that the chicken introns, K7 to K11, should also have existed in the progenitor GAPDH gene. Therefore, among a total of 18 intron positions in four GAPDH genes, 15 of them should have existed in the progenitor GAPDH gene. Whether the remaining three introns (B6, C6, and C8) are the result of deletional or insertional events remains to be elucidated.

In summary, we have provided evidence that some GAPDH introns existed before the divergence of prokaryotes and eukaryotes, consistent with the idea that introns played a role in the assembly of the progenitor GAPDH gene. In addition, a comparison of the intron positions with the structural domains of GAPDH indicates that the majority, if not all, of the introns that exist in the four GAPDH genes whose sequences are known should have existed in the progenitor GAPDH gene.

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HIV-Infected Cells Are Killed by rCD4-Ricin A Chain

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The gp120 envelope glycoprotein of the human immunodeficiency virus (HIV), which is expressed on the surface of many HIV-infected cells, binds to the cell surface molecule CD4. Soluble derivatives of recombinant CD4 (rCD4) that bind gp120 with high affinity are attractive vehicles for targeting a cytotoxic reagent to HIV-infected cells. Soluble rCD4 was conjugated to the active subunit of the toxin ricin. This conjugate killed HIV-infected H9 cells but was 1/1000 as toxic to uninfected H9 cells (which do not express gp120) and was not toxic to Daudi cells (which express major histocompatibility class II antigens, the putative natural ligand for cell surface CD4). Specific killing of infected cells can be blocked by rgp120, rCD4, or a monoclonal antibody to the gp120 binding site on CD4.

MOST INDIVIDUALS INFECTED with the human immunodeficiency virus (HIV) develop acquired immunodeficiency syndrome (AIDS) (*1*), which is characterized by the progressive depletion of T cells expressing CD4, the cellular receptor for HIV (*2*). A potential approach for preventing or delaying the onset of AIDS is to eliminate cells producing viral proteins early in the course of the disease. This may prevent the spread of infection and the release of viral proteins that may participate in the pathogenesis of the disease (*3*). HIV-infected cells could be eliminated with a toxic agent coupled to a targeting molecule that would bind only to cells expressing HIV-encoded proteins. An attractive targeting entity is recombinant soluble CD4 (rCD4) (*4*, *5*) that binds to gp120, the envelope glycoprotein of HIV, with an affinity comparable to that of cell surface CD4 (*4*). Although gp120, which is expressed on the surface of many HIV-infected cells, shows extensive variability among different strains of HIV, its CD4 binding site is highly conserved (*6*).

Conjugates of toxins and cell-reactive ligands can specifically delete cells in vitro and in vivo (*7*). We and others have used the A chain of the plant toxin, ricin, conjugated to cell-reactive antibodies. Such conjugates kill cells after endocytosis of the conjugate-antigen complex and translocation of the A chain into the cytosol where it inhibits protein synthesis (*7*). Below we describe the coupling of soluble rCD4 to deglycosylatec

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